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Patentanmeldung Nr. Patent application No. Demande de brevet n°

03103580.1

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Anmeldung Nr:
Application no.: 03103580.1
Demande no:

Anmeldetag:
Date of filing: 26.09.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Applied Research Systems ARS Holding N.V.
Pietermaai 15
Curacao
ANTILLES NEERLANDAISES

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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NOVEL LEADER SEQUENCES FOR USE IN PRODUCTION OF PROTEINS

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N15/00

Am Anmeldetag benannte Vertragsstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT RO SE SI SK TR LI

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NOVEL LEADER SEQUENCES FOR USE IN PRODUCTION OF PROTEINS

FIELD OF THE INVENTION

5 This invention relates to leader sequences for production of proteins. More specifically, the invention relates to DNA constructs encoding leader sequences comprising an immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide. The invention further relates to the use of these DNA constructs for producing proteins in
10 mammalian cells.

BACKGROUND

1. Processing of protein precursors

15 Secreted proteins are expressed initially inside the cell in a precursor form containing a leader sequence ensuring entry into the secretory pathway. Such leader sequences, named signal peptides, direct the expressed product across the membrane of the endoplasmic reticulum (ER). Signal peptides are generally cleaved off by signal peptidases during translocation to the ER. Once entered in the secretory pathway, the protein is transported to
20 the Golgi apparatus. From the Golgi the protein can follow different routes that lead to compartments such as the cell vacuole or the cell membrane, or it can be routed out of the cell to be secreted to the external medium (Pfeffer and Rothman (1987) Ann.Rev.Biochem. 56:829-852).

For industrial production of a secreted protein, the protein to be produced needs to be
25 secreted efficiently from the host cell or the host organism. The signal peptide may be, e.g., the native signal peptide of the protein to be produced, a heterologous signal peptide, or a hybrid of native and heterologous signal peptide. Numerous signal peptides are used for production of secreted proteins. One of them is a murine immunoglobulin signal peptide (IgSP, EMBL Accession No. M13331). IgSP was first identified in 1983 by Loh et al. (Cell. 33:85-93).
30 IgSP is known to give a good expression in mammalian cells. For example, EP patent No. 0382762 discloses a method of producing horseradish peroxidase by constructing a fusion polypeptide between IgSP and horseradish peroxidase.

However, several problems are encountered with the use of currently known signal peptides. One problem often encountered when producing a human protein from a non-human
35 host cell or organism is that the native signal peptide does not ensure efficient translocation and/or cleavage of the signal peptide. This leads to low rates of protein secretion and/or to secretion of mature proteins that display N-terminal extensions due to an incorrect cleavage of

the signal peptide. Thus the choice of the signal peptide is of great importance for industrial production of a protein.

In addition of leader sequences directing the secretion of the protein, a precursor form can comprise supplemental leader sequences that are cleaved during maturation. These supplemental leader peptides, named propeptides, usually follow the signal peptide. Virtually all peptide hormones, numerous bioactive proteins (for example, growth factors, receptors and cell-adhesion molecules), and many bacterial toxins and viral envelope glycoproteins comprise a propeptide that is post-translationally excised to generate the mature and biologically active protein (Seidah and Chretien (1999) *Brain Res.* 848:45-62).

Propeptides are cleaved off by enzymes named proprotein convertases. Mammalian proprotein convertases include, e.g., the subtilisin convertases PCSK1, PCSK2 and furin. Furin is ubiquitously expressed and located in the trans-Golgi network. Furin proteolytically activates large numbers of proproteins substrates in secretory pathway compartments. (Thomas (2002) *Nat Rev Mol Cell Biol.* 3:753-766). More specifically, furin localizes to the Trans Golgi Network – a late Golgi structure that is responsible for sorting secretory pathway proteins to their final destinations, including the cell surface, endosomes, lysosomes and secretory granules. The site that furin cleaves has been extensively studied. The cleavage site is positioned after the carboxyl-terminal arginine of the consensus sequence R-X-L/R-R, wherein X may represent any amino acid (Nakayama (1997) *Biochem. J* 327:625-635). The cleavage efficiency is increased when X is a lysine, a valine, an isoleucine or an alanine (Watanabe et al (1992) *J Biol. Chem.* 267:8270-8274).

2. The tissue-type plasminogen activator precursor

The human tissue-type plasminogen activator precursor (tPA, SwissProt Accession No. P00750) is synthesized as a precursor form of 562 amino acids comprising a leader sequence of 35 amino acids. This leader sequence comprises a signal peptide of 23 amino acids followed by a propeptide of 12 amino acids.

Köhne et al. (1999, *J. Cell. Biochem.* 75 :446-461) showed that the tPA leader sequence of 35 amino acids was able to rescue intracellular transport of a chimeric Tumor Necrosis Factor Receptor - Immunoglobulin protein (TNFR-Ig) in which all N-linked glycosylation sites had been deleted. In 1999, Etcheverry et al. reported that a leader sequence of 13 amino acids, which comprised the last amino acid of the signal peptide and the entire propeptide of tPA, was able to enhance secretion of a TNFR-Ig fusion when inserted between the TNFR native signal peptide and the TNFR-Ig polypeptide (Etcheverry et al., ESACT meeting, abstract O1.07/P1.02).

Thus protein processing is a fundamental process for efficient protein secretion, and the choice of the leader sequence is a critical step when producing a secreted polypeptide. In many cases, the leader sequence leads to a low level of secretion or no secretion at all, or to an incorrect or incomplete proteolytic processing. It is therefore the object of the present invention to provide leader sequences that ensure a more efficient secretion and/or processing of polypeptides.

SUMMARY OF THE INVENTION

10 The present invention is based on the finding that a leader sequence comprising an immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide allows a more efficient secretion and processing of proteins of interest than other known leader sequences. In addition, it has been found that a leader sequence comprising a truncated form of the human tPA propeptide, wherein the carboxyl-terminal extremity of the tPA propeptide
15 consists of amino acids Arg-Xaa-Arg-Arg, allows an efficient secretion and processing of proteins of interest.

Therefore, a first aspect of the invention relates to a DNA construct comprising a sequence encoding an IgSP-tPA pre-propeptide comprising an immunoglobulin signal peptide fused to a tPA propeptide.

20 A second aspect relates to A DNA construct comprising a sequence encoding a human tissue-type plasminogen activator propeptide (tPA) wherein the carboxyl-terminal extremity of said tPA propeptide consists of amino acids Arg-Xaa-Arg-Arg

A third aspect relates to a host cell transformed with a DNA construct according to the invention.

25 A fourth aspect relates to a process for the production of a polypeptide of interest comprising the step of transfecting a host cell with a DNA construct in accordance with the invention.

A fifth aspect relates to a process for the production of a polypeptide of interest comprising the step of culturing a host cell of the invention .

30 A sixth aspect relates to the use of a DNA construct in accordance with the invention for producing a polypeptide of interest.

A seventh aspect relates to a fusion polypeptide encoded by a DNA construct in accordance with the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an alignment between an IgSP pre-propeptide in accordance with the invention (SEQ ID NO: 1) and the native tPA pre-propeptide (SEQ ID NO: 2).

Figure 2 shows a scheme of the pGL3-GH-TBP-1380 vector used to construct the different signal peptide fused to the TBPI protein.

Figure 3 shows a scheme of the pEF1-GH-TBP-1403 vector used to express the leader peptides-TBPI fusion proteins in transient transfection assays.

Figure 4 shows the amount of TBPI protein detected in supernatant versus cytoplasm of cells transfected with the indicated constructs. Lane GH_SP: TBPI fused to the Growth hormone signal peptide; Lane SEAP_SP: TBPI fused to the secreted alkaline phosphatase signal peptide; Lane IgSP: TBPI fused to the murine immunoglobulin IgG μ -heavy chain signal peptide; Lane IgSP-tPA: TBPI fused to an IgSP pre-propeptide in accordance with the invention.

Figure 5 corresponds to a scheme of the CMV-UbB-LUC-1433 vector used to express the leader peptides-TBPI fusion proteins in stable transfection assays.

Figure 6 shows the amount of TBPI protein detected in the supernatant of pools of clones transfected with IgSP-tPA or with tPA-tPA pre-propeptides fused to TBPI. Pools were maintained either in puromycin and neomycin co-selection (neo/puro) or in puromycin minus Hypoxanthine-Thymidine co-selection (HT/puro). Open box and dark box represent two different pulses of 48hrs at 37 °C in medium with 10% FCS. Stripped or squared box represent two pulses of 48hrs at 32 °C in serum-free medium.

BRIEF DESCRIPTION OF THE SEQUENCES OF THE SEQUENCE LISTING

SEQ ID NO: 1 corresponds to the protein sequence of an IgSP-tPA pre-propeptide in accordance with the invention.

SEQ ID NO: 2 corresponds to the protein sequence of the human tPA pre-propeptide.

SEQ ID NO: 3 corresponds to the protein sequence of the murine IgG μ -heavy chain signal peptide.

SEQ ID NO: 4 corresponds to the protein sequence of the human growth hormone signal peptide.

SEQ ID NO: 5 corresponds to the protein sequence of the human secreted alkaline phosphatase signal peptide.

SEQ ID NO: 6 corresponds to the nucleic sequence of an IgSP-tPA pre-propeptide in accordance with the invention.

SEQ ID NO: 7 corresponds to the nucleic sequence of the human tPA pre-propeptide.

SEQ ID NO: 8 corresponds to the nucleic sequence of the murine IgG μ -heavy chain signal peptide.

SEQ ID NO: 9 corresponds to the protein sequence of the soluble portion of the TNF receptor p55.

SEQ ID NO: 10 corresponds to the protein sequence of mature interferon gamma receptor chain.

5 SEQ ID Nos. 11 to 20 correspond to primers used to construct and amplify the Ig μ -heavy chain signal peptides.

SEQ ID NO: 21 to 34 correspond to primers used to construct and amplify the human growth hormone signal peptide.

10 Seq ID NO: 35 to 41 correspond to primers used to construct and amplify the human secreted alkaline phosphatase signal peptide.

Seq ID NO: 42 to 49 correspond to primers used to construct and amplify the IgSP-tPA pre-propeptide in accordance with this invention.

Seq ID NO: 50: correspond to the nucleic sequence of the soluble extracellular portion of the p55 Tumor necrosis factor.

15 Seq ID NO: 51 to 54 correspond to primers used to introduce a deletion of three amino acids into the IgSP-tPA and tPA pre-propeptides in accordance with this invention.

Seq ID NO: 55 to 58 correspond to primers used to generate the tPA-pre-propeptide.

DETAILED DESCRIPTION OF THE INVENTION

20 The present invention stems from the finding that leader sequences comprising an immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide allow a more efficient secretion and processing of proteins than other known leader sequences. As shown in examples 2 and 3, leader sequences of the present invention are at least 2 fold more efficient in promoting secretion of proteins of interest than prior art leader sequences.

25 In addition, it has been found that a leader sequence comprising a truncated form of the human tPA propeptide, wherein the carboxyl-terminal extremity of the tPA propeptide consists of amino acids Arg-Xaa-Arg-Arg, allows an efficient secretion and processing of proteins of interest.

30 Accordingly, the present invention provides novel leader sequences comprising (i) an immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide; or (ii) a tPA propeptide wherein its carboxyl-terminal extremity consists of amino acids Arg-Xaa-Arg-Arg. The use of these leader sequences for producing proteins of interest in mammalian cells is a further aspect of the present invention.

35 A first aspect of the present invention relates to a DNA construct comprising a sequence encoding an IgSP-tPA pre-propeptide comprising an immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide.

As used herein, the term "signal peptide" refers to a leader sequence ensuring entry into the secretory pathway. As used herein, the term "propeptide" refers to a leader sequence that follows a signal peptide. As used herein, the term "pre-propeptide" refers to a leader sequence comprising a signal peptide and a propeptide. As used herein, the term "leader sequence" refers to a sequence located at the amino terminal end of the precursor form of a protein. Leader sequences are cleaved off during maturation.

As further used herein, the term "IgSP-tPA pre-propeptide" refers to a leader sequence according to the present invention that comprises an immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide.

As shown in example 1, IgSP-tPA pre-propeptides ensures a more efficient secretion of the soluble portion of the TNF receptor p55 (TBPI) than various signal peptides fused directly to TBPI. Example 2 demonstrates that the IgSP-tPA pre-propeptide is more efficient in promoting TBPI secretion than a tPA pre-propeptide alone. Example 3 demonstrates that the IgSP-tPA pre-propeptide is more efficient in promoting secretion of the mature interferon gamma receptor chain (IFNAR) than the native IFNAR signal peptide. Accordingly, IgSP-tPA pre-propeptides ensure a more efficient secretion of polypeptides of interest than any known leader sequence.

Numerous immunoglobulin (Ig) signal peptides from different species are known and are all encompassed within the scope of the present invention. In a preferred embodiment, the Ig signal peptide is a murine immunoglobulin signal peptide. Preferably, the murine Ig signal peptide is a murine IgG μ -heavy chain signal peptide of SEQ ID NO: 3.

The DNA construct of the present invention comprises a sequence encoding a tissue-type plasminogen activator (tPA) propeptide of any origin. For example, the DNA construct can comprise a sequence encoding a tPA propeptide of human, murine, rat or bovin origin. Preferably, the DNA construct comprises a sequence encoding a tPA propeptide of human origin.

In a further embodiment, the DNA construct of the present invention comprises a sequence encoding a human tPA propeptide, the carboxyl-terminal extremity of said tPA propeptide consisting of amino acids Arg-Xaa-Arg-Arg. Such a propeptide corresponds to a truncated propeptide lacking the three carboxyl-terminal amino acids of the native human tPA propeptide (see Figure 1). Preferably, the human tPA propeptide consists of amino acids 24 to 32 of SEQ ID NO: 2.

In a preferred embodiment, the DNA construct of the present invention encodes a pre-propeptide comprising SEQ ID NO: 1. In a most preferred embodiment, the pre-propeptide consists of SEQ ID NO: 1.

The terms "comprising", "consisting of", or "consisting essentially of" have distinct meanings. However, each term may be substituted for another herein to change the scope of the invention.

As used herein, the expression "a polypeptide A fused to a polypeptide B" refers to a fusion polypeptide comprising the sequences of polypeptides A and B, wherein the sequence of polypeptide A is located at the amino-terminal extremity of the sequence of polypeptide B within said fusion polypeptide. The term "fused to", as used herein, is not limited to a direct fusion of polypeptides. For example, the cloning strategy may lead to the presence of amino acids between polypeptides A and B. However, a direct fusion of polypeptides is preferred. Methods of constructing DNA constructs comprising sequences encoding fusion polypeptides are well known in the art. For example, methods described in examples 1 to 3 may be used.

Another preferred embodiment of the present invention relates to a DNA construct encoding a fusion polypeptide comprising an IgSP -tPA pre-propeptide fused to a polypeptide of interest.

In accordance with the present invention, the polypeptide of interest may be any polypeptide for which production is desired. For example, the polypeptide of interest may be, e.g., a naturally secreted protein, a normally cytoplasmic protein, a normally transmembrane protein, or a human or a humanized antibody. When the protein of interest is a normally cytoplasmic or a normally transmembrane protein, the protein has preferably been engineered in order to become soluble. The polypeptide of interest may be of any origin. Preferred polypeptides of interest are of human origin.

In preferred embodiments, the polypeptide is selected from the group consisting of chorionic gonadotropin, follicle-stimulating hormone, lutropin-choriogonadotropic hormone, thyroid stimulating hormone, human growth hormone, interferons (e.g., interferon beta-1a, interferon beta-1b), interferon receptors (e.g., interferon gamma receptor), TNF receptors p55 and p65, interleukins (e.g., interleukin-2, interleukin-11), interleukin binding proteins (e.g., interleukin-18 binding protein), anti-CD11a antibodies, and muteins, fragments, soluble forms, functional derivatives, fusion proteins thereof.

Other preferred polypeptides of interest include, e.g., erythropoietin, granulocyte colony stimulating factor, granulocyte-macrophage colony-stimulating factor, pituitary peptide hormones, menopausal gonadotropin, insulin-like growth factors (e.g., somatomedin-C), keratinocyte growth factor, glial cell line-derived neurotrophic factor, thrombomodulin, basic fibroblast growth factor, Insulin, Factor VIII, somatropin, bone morphogenetic protein-2, platelet-derived growth factor, hirudin, epoetin, recombinant LFA-3/IgG1 fusion protein,

glucocerebrosidase, and muteins, fragments, soluble forms, functional derivatives, fusion proteins thereof.

5 A second aspect of the present invention is directed to a DNA construct comprising a sequence encoding a human tissue-type plasminogen activator propeptide characterized in that its carboxyl-terminal extremity consists of amino acids Arg-Xaa-Arg-Arg.

In a first embodiment, the tPA propeptide of the present invention is a human tPA propeptide consisting of amino acids 24 to 32 of SEQ ID NO: 2.

10 In a second embodiment, the DNA construct comprising a tPA propeptide in accordance with the invention further comprises a signal sequence fused to said tPA propeptide.

Any signal peptide currently used in the art for promoting protein secretion may be used in the above embodiment. Such signal peptides include, e.g., the human growth hormone signal peptide (see, e.g., EP application 01 999 652.9), the secretion competent
15 polypeptide disclosed in EP application 00 906 103.7, the human erythropoietin signal peptide, the human albumin signal peptide, the human secreted alkaline phosphatase signal peptide and the rotavirus VP7 glycoprotein signal peptide.

In a third embodiment, the DNA comprising a tPA propeptide in accordance with the invention encodes a fusion polypeptide comprising said tPA propeptide fused to a polypeptide
20 of interest.

In a preferred embodiment, the DNA construct comprising a sequence encoding an IgSP-tPA pre-propeptide and/or a tPA propeptide in accordance with the invention is included in a vector.

25 The term "vector" refers to any carrier of exogenous DNA or RNA that is useful for transferring exogenous DNA to a host cell for replication and/or appropriate expression of the exogenous DNA by the host cell.

In a further preferred embodiment, the vector is an expression vector. An "expression vector" comprises appropriate signals that drive expression in host cells of a polynucleotide
30 inserted in said vector. Preferably, the polynucleotides inserted in said vector encode a polypeptide of interest. The appropriate signals include various regulatory elements, such as enhancers and/or promoters from both viral and mammalian sources. Selectable markers for establishing permanent, stable cell clones expressing the products such as, e.g., a dominant drug selection, are generally included in the expression vectors of the invention, as they are
35 elements that link expression of the drug selection markers to expression of the polypeptide.

In a further preferred embodiment, the vector is a gene activation vector. The gene activation technology is a technology allowing the production of proteins of interest without introducing the gene or cDNA of interest into the host cell (see e.g., EP application 91 903 051.0). For example, the gene activation technology may bypass regulatory DNA sequences set in the "off position" with regulatory DNA sequences set in the "on position" in order to activate the gene of interest. A "gene activation vector" comprises appropriate signals that drive expression in host cells of a polynucleotide present in said host cell.

In a further preferred embodiment, the vector is a gene therapy vector. Expression vectors that may be used for gene therapy are well known in the art. Preferably, the gene therapy vector is a lentiviral derived vector, which has been shown to be very efficient in the transfer of genes, in particular within the CNS. Other well-established viral vectors, such as adenoviral derived vectors, may also be used according to the invention.

A third aspect of the invention relates to a host cell transformed with a DNA construct according to the invention. Many host cells are suitable in accordance with the present invention, such as primary or established cell lines from a wide variety of eukaryotes including plant and animal cells. Preferably, said host cell is an eukaryotic cell. Most preferably, said host cell is a mammalian cell.

For example, suitable host cells include CHO cells, COS cells, CV1 cells, mouse L cells, HT1080 cells, BHK-21 cells, HEK293 cells, NIH-3T3 cells, LM cells, Y1 cells, NS0 and SP2/0 mouse hybridoma cells and the like, Namalwa cells, RPMI-8226 cells, Vero cells, WI-38 cells, MRC-5 cells or other immortalized and/or transformed cells.

Preferably, the host cell is a CHO cell, and more preferably a CHO-S cell, described e.g. by Shotwell et al. (1982, J Biol. Chem. 257:2974-2980).

In a fourth aspect, the invention relates to a process for the production of a polypeptide of interest comprising the step of transfecting a host cell with a DNA construct according to the invention.

In a fifth aspect, the invention relates to a process for the production of a polypeptide of interest comprising the step of culturing a host cell in accordance with the invention.

Such processes according to the invention lead to secretion of the protein of interest, which may be harvested from the cell culture supernatant. Depending on the intended use, the cell synthesizing the polypeptide may be the product of the process according to the invention.

In a preferred embodiment, these processes further comprise the step of isolating the polypeptide of interest from the host cells. This step is particularly advantageous and easy to

carry out since the protein of interest may simply be isolated from the cell culture supernatant.

These processes may be used in transient, stable, episomal or viral expression systems. In a preferred embodiment, the transfection is stable transfection.

5

In a sixth aspect, the DNA construct according to the invention is used for producing a polypeptide of interest.

A seventh aspect of the invention relates to fusion polypeptides comprising an IgSP-tPA pre-propeptide and/or a tPA propeptide according to the invention fused to a polypeptide of interest.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished patent application, issued patents or any other references, are entirely incorporated by reference herein, including all data, tables, figures and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various application such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or

terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

5

EXAMPLES

Example 1: Comparison between the IgSP-tPA pre-propeptide and the human growth hormone signal peptide, the secreted alkaline phosphatase signal peptide, the murine immunoglobulin signal peptide.

10

1.1. Constructions.

1.1.1 IgSP

The murine IgG μ -heavy chain signal peptide of SEQ ID NO: 3 (IgSP) cloned as follows. Primers of Seq ID Nos. 13 to 20 were incubated with the T4 polynucleotide kinase (Stratagene) for 2h30 at 37°C, and heat inactivated at 75°C for 10 min. The treated primers were ligated using cycle ligation with Pfu Ligase from Stratagene as recommended by the manufacturer in the following cycle conditions:

- 95°C for 1min;
- 40 cycles at 95°C for 30", 57°C for 90", 70°C for 2min
- 70°C for 10min.

20

The annealed oligos were then purified with QIAquick columns, and PCR amplified with PFU turbo using standard conditions with the primer SEQ ID No 13 and 17 under the following PCR conditions:

- 95°C 5min
- 30 cycles of 95 °C for 45", 70 °C for 45"; and
- 70 °C for 10min.

25

The PCR product was purified and digested with Bgl-II and BsrGI and cloned into the pGL3-GH-TBPI-1380 vector (Figure 2). The product was sequenced and was found to reflect the expected sequence.

30

1.1.2 GH_SP

The human growth hormone signal peptide of SEQ ID NO: 4 (GH_SP) was cloned as follows using primers of SEQ ID No. 21 to 34. The primers were annealed as described above. At the end of the annealing, the product was purified and re-amplified by PCR using the same conditions as above. The PCR product was re-amplified using primers of SEQ ID

35

Nos. 21 and 34. The product was then cloned into the pGL3 -GH-TBPI380 vector at the Bgl-II and BsrGI cloning sites.

1.1.3. SEAP_SP

5 The human secreted alkaline phosphatase signal peptide of SEQ ID NO: 5 (SEAP_SP) was cloned using primers of SEQ ID No. 35 to 41. The primers were annealed as described above. At the end of the annealing, the product was purified and re-amplified by PCR using the same conditions as above. The PCR product was re-amplified using primers of SEQ ID Nos. 35 and 40. The product was then cloned into the pGL3 -GH-TBPI380 vector at the Bgl-II and BsrGI cloning sites.

10 1.1.4. IgSP-tPA

The studied IgSP-tPA pre-propeptide comprised: (i) the murine IgG μ -heavy chain signal peptide fused to (ii) a truncated tPA propeptide that lacks the three carboxyl-terminal amino acids of the native tPA propeptide. This IgSP-tPA pre-propeptide is shown as SEQ ID NO: 1. This IgSP-tPA pre-propeptide was cloned as follows.

15 Primers of SEQ ID No. 42 to 49 were annealed as described above. At the end of the annealing, the product was purified and re-amplified by PCR using primers of SEQ ID 45 and 49. The purified PCR product was then cloned into the pGL3 -GH-TBPI380 vectors at the Bgl-II and BsrGI cloning sites.

20 A recombinant PCR was performed for deleting three amino-acid (GAR) from the known tPA propeptide. The 5' end of the construct was PCR amplified with primers of SEQ ID Nos. 51 and 52, and the 3' end was PCR amplified with primers of SEQ ID Nos. 53 and 54. The full-length IgSP-tPA-TBPI construct was then PCR amplified with primers of SEQ ID Nos. 51 and 54 and the two recombinant products obtained from the previous PCR.

1.1.5. Fusion of the above leader sequences to TBPI

25 The above leader sequences were fused to the soluble portion of the TNF receptor p55 protein (TBPI, SEQ ID NO: 50). The pEF1-GH-TBPI-1403 vector (Figure 3), which allows expression of TBPI under the Human Elongation Factor 1 (EF1) promoter, was digested by Nco-I and Xba-I. Fragments encoding the leader sequences were subcloned into pEF1-GH-TBPI-1403 as Nco-I or as Bsa-I -Xba-I fragments. The resulting IgSP-TBPI, GH_SP-TBPI
30 SEAP_SP-TBPI and IgSP-tPA constructs were sequenced and were found to reflect the expected sequence.

1.2. Measurement of protein secretion

Each construct was transfected in CHO-DUKX-B11 cells using standard lipid mediated transfection as described in standard laboratory manuals (Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press). 48hrs after transfection, medium and cells were harvested. Cells were washed twice in PBS and the pellet was lysed on ice using 300µl of Cytobuster buffer (Novagen) in the presence of a protease inhibitor cocktail (Roche). Cellular debris were spun down by centrifugation at 16 000 g for 10 mn at 4 °C. The supernatant was harvested and kept at -20 °C before being processed. The amount of TBPI released in the supernatant or in intracellular compartment was analyzed by an ELISA. The relative expression of TBPI was measured and reported.

The results of the experiment are shown on Figure 4. The IgSp-tPA signal propeptide is able to boost secretion of TBPI from cells as demonstrated by the increased amount of TBPI detected in the supernatant versus the amount of TBPI detected in intracellular compartments. Thus the IgSP-tPA-TBPI construct, comprising TBPI fused to an IgSP-tPA propeptide, increases secretion of TBPI compared to the constructs corresponding to the TBPI protein fused to the IgSP signal peptide, to the secreted alkaline phosphatase signal peptide or to the growth hormone signal peptide.

Accordingly, the IgSP-tPA pre-propeptide ensures a more efficient secretion of TBPI than any other signal peptide fused directly to TBPI without propeptide.

Example 2: Comparison between the IgSP-tPA pre-propeptide and the tPA pre-propeptide.

2.1. Constructions.

2.1.1. *IgSP-tPA-TBPI*

The IgSP-tPA-TBPI construct described in 1.1.5. was digested by Xba-I. The fragment comprising IgSP-tPA-TBPI was cloned into the pmCMV-UbB-LUC-1433 expression vector (Figure 5) digested by Nco-I and Xba-I.

2.1.2. *tPA-TBPI*

A tPA pre-propeptide of SEQ ID NO: 2 comprising: (i) the tPA signal peptide and (ii) a truncated tPA propeptide that lacks the three carboxyl-terminal amino acids of the native tPA propeptide was generated as follows.

The human tPA pre-propeptide was cloned using the IgSP-tPA-TBPI construct as a template. A first PCR was performed with primers of SEQ ID No 55 and 56 in order to amplify the tPA propeptide and the 5' end of TBPI. In a second PCR, the PCR product from the first step was extended by re-amplification with primers of SEQ ID Nos. 57, 58 and 56. The PCR

product was then cloned into the pGL3-GH-TBPI-1380 vector (Figure 2) digested with Bgl-II and BsrGI. A recombinant PCR was performed as described in 1.1.4. with primers of SEQ ID Nos. 51 to 54 in order to introduce an internal deletion of three amino-acid (GAR) into the known tPA propeptide.

- 5 The resulting tPA-TBPI construct was digested by a Bsa-I and Xba-I. The fragment comprising tPA-TBPI was cloned into the pmCMV-UbB-LUC-1433 expression vector digested with Nco-I and Xba-I.

2.2. Measurement of protein secretion

- 10 CHO cells were transfected using lipofectamine with the IgSP-tPA-TBPI and the tPA-TBPI constructs. In one series of experiments, the TBPI expression vector was co-transfected with SV40neo and puro vectors for selection. In another series, the TBPI expression vector co-transfected with SV40dhfr and SV40 puro vectors. Pools of stable expressing clones representing at least 100 clones were expanded in different selection medium (600 $\mu\text{g}.\text{ml}^{-1}$
- 15 neomycin, 6 $\mu\text{g}.\text{ml}^{-1}$ puromycin, or HT + 6 $\mu\text{g}.\text{ml}^{-1}$ puromycin). Pools were split and cells seeded either in FCS-containing or in serum-free medium. The media were harvested after 48 hrs, and the amount of TBPI released in the supernatant was determined using an ELISA. The result of this experiment is shown in Figure 6. Each box represents a pool. Open boxes and dark boxes represent two different pulses in 10% FCS-containing medium at 37 °C. Striped or
- 20 squared box represent two different pulses in serum-free medium at 32 °C. The initial number of seeded cells and the pulse periods were similar in each experiment.

- Figure 6 shows that in all conditions that were studied, pools of IgSP-tPA-TBPI expressing cells had higher titers of TBPI than pools of tPA-TBPI expressing cells. The results clearly indicate that the IgSP-tPA construct is at least two fold better than tPA construct in
- 25 terms of quantity of secreted protein that is produced.

Accordingly, the novel combination of the tPA propeptide with the IgSP signal peptide is more efficient at promoting secretion of proteins than the tPA pre-propeptide.

- In addition, the sequence of the N-terminal extremity of the TBPI protein secreted from
- 30 IgSP-tPA-TBPI expressing cells was determined by N-terminal sequencing using Edman degradation. It was found that 100% of the proteins had been cleaved after the last arginine residue of the tPA. Thus the IgSP-tPA pre-propeptide ensures an efficient and reliable processing of polypeptides.

Example 3: Comparison between the IgSP-tPA pre-propeptide and the Interferon gamma receptor signal peptide for production of Interferon gamma .

5 **3.1. Constructs.**

The IgSP pre-pro peptide was fused to a mature Interferon gamma receptor chain protein (IFNAR) and cloned into (i) the mCMV-UbB-LUC-1433 vector (Figure 3); or (ii) a vector comprising the promoter of the mCMV-IE2 gene described in EP application 03 100 617.4 .

10 A full-length IFNAR, comprising the native signal peptide, was cloned into (i) the mCMV-UbB-LUC-1433 vector; or (ii) the expression vector comprising the promoter of the mCMV-IE2 gene described in EP application 03 100 617.4.

3.2. Measurement of protein secretion

3.2.1. Protocol

15 Constructs were transfected into CHO cells using standard lipid mediated transfection protocols. The secreted proteins were harvested after 48 hrs. A specific Elisa test was used to quantify the amount of IFNAR secreted in the supernatant. The transfections were normalized with a luciferase construct co-transfected with the IFNAR vector. A standard luciferase assay was used as described in standard laboratory manuals (Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press).

3.2.1. Constructs comprising the CMV vector

The IgSP-tPA pre-propeptide was about 2.3 fold more efficient in promoting secretion of the IFNAR protein in the supernatant than the native IFNAR signal peptide.

3.2.2. Constructs comprising the promoter of the mCMV-IE2 gene.

25 The IgSP-tPA pre-propeptide was about 4.3 fold more efficient in promoting secretion of the IFNAR protein in the supernatant than the native IFNAR signal peptide.

REFERENCES

- Etcheverry et al., ESACT meeting, abstract O1.07/P1.02
- Köhne et al (1999) J. Cell. Biochem. 75 :446-461
- 5 Loh et al. (1983) Cell. 33:85-93
- Nakayama (1997) Biochem. J 327:625-635
- Pfeffer and Rothman (1987) Ann.Rev.Biochem. 56:829-852
- Seidah and Chretien (1999) Brain Res. 848:45-62
- Shotwell et al. (1982) J Biol. Chem. 257:2974-2980
- 10 Thomas (2002) Nat Rev Mol Cell Biol. 3:753-766
- Watanabe et al (1992) J Biol. Chem. 267:8270-8274

CLAIMS:

1. A DNA construct comprising a sequence encoding an IgSP-tPA pre-propeptide comprising an immunoglobulin signal peptide fused to a tissue-type plasminogen activator (tPA) propeptide.
2. The DNA construct of claim 1, wherein said immunoglobulin signal peptide is a murine immunoglobulin signal peptide.
3. The DNA construct of claim 2, wherein said murine immunoglobulin signal peptide comprises SEQ ID NO: 3.
4. The DNA construct of any of claims 1 to 3, wherein said tPA propeptide is a human tPA propeptide, the carboxyl-terminal extremity of said tPA propeptide consisting of amino acids Arg-Xaa-Arg-Arg.
5. The DNA construct of claim 4, wherein said tPA propeptide consists of amino acids 24 to 32 of SEQ ID NO: 2.
6. The DNA construct of any of claims 1 to 5, wherein said pre-propeptide comprises SEQ ID NO: 1.
7. The DNA construct of any of claims 1 to 6, wherein said DNA construct encodes a fusion polypeptide comprising said IgSP-tPA pre-propeptide fused to a polypeptide of interest.
8. A DNA construct comprising a sequence encoding a human tissue-type plasminogen activator propeptide (tPA) wherein the carboxyl-terminal extremity of said tPA propeptide consists of amino acids Arg-Xaa-Arg-Arg.
9. The DNA construct of claim 8, wherein said tPA propeptide consists of amino acids 24 to 32 of SEQ ID NO: 2.
10. The DNA construct of claim 8 or 9, further comprising a signal sequence fused to said tPA propeptide.
11. The DNA construct of any of claims 8 to 10, wherein said DNA construct encodes a fusion polypeptide comprising said tPA propeptide fused to a polypeptide of interest.
12. The DNA construct of any of claims 1 to 11, wherein said DNA construct is included in a vector.
13. The DNA construct of any of claims 12, wherein said vector is an expression vector.

14. The DNA construct of any of claims 12, wherein said vector is a gene activation vector.
15. A host cell transformed with the DNA construct of any of claims 1 to 14.
16. The host cell of claim 15, wherein said cell is selected from the group consisting of CHO cell, a COS cell, a CV1 cell, a mouse L cell, a HT1080 cell, a BHK cell, a HEK29 cell, , a NIH-3T3 cell, a LM cell and a YI cell, NS0 and SP2/0 mouse hybridoma and the like, Namalwa, RPMI-8226, Vero, WI-38, MRC-5 and the like.
17. The host cell of claim 16, wherein said cell is a CHO cell.
18. A process for the production of a polypeptide of interest comprising the step of transfecting a host cell with the DNA construct of any of claims 1 to 14.
19. A process for the production of a polypeptide of interest comprising the step of culturing the host cell of any of claims 15 to 17.
20. The process of claim 18 or 19, further comprising the step of isolating the polypeptide of interest from said host cells.
21. The process of any of claims 18 to 20, wherein the transfection is stable transfection.
22. Use of the DNA construct of any of claims 1 to 14 for producing a polypeptide of interest.
23. A fusion polypeptide encoded by the DNA constructs of claim 7 or 11.

ABSTRACT

This invention encompasses novel leader sequences for production of proteins. More specifically, the invention relates to DNA constructs encoding leader sequences comprising an immunoglobulin signal peptide fused to a tissue-type plasminogen activator propeptide, and to DNA constructs encoding leader sequences comprising a truncated human tissue-type plasminogen activator propeptide. The invention further relates to the use of these DNA constructs for producing proteins in mammalian cells.

FIGURE 1

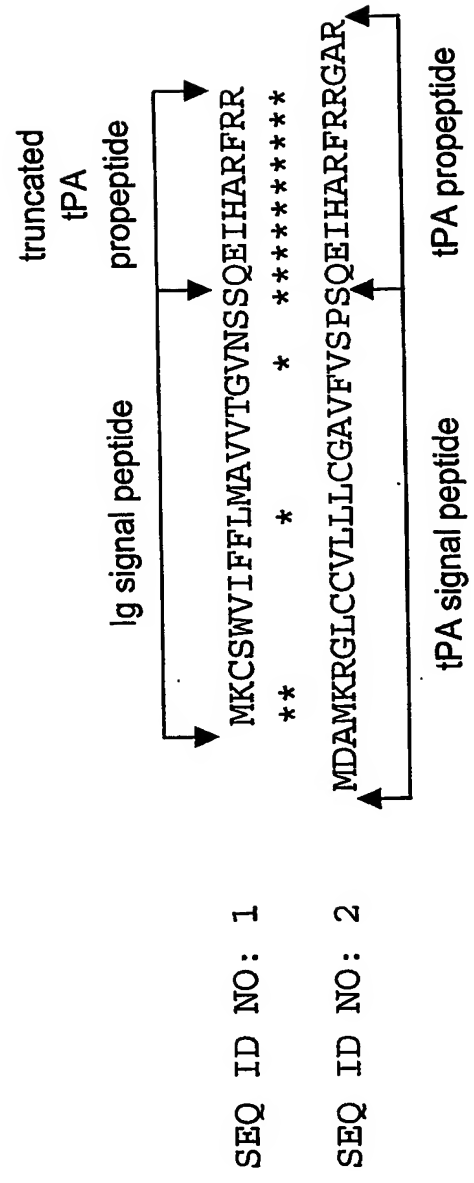


FIGURE 2

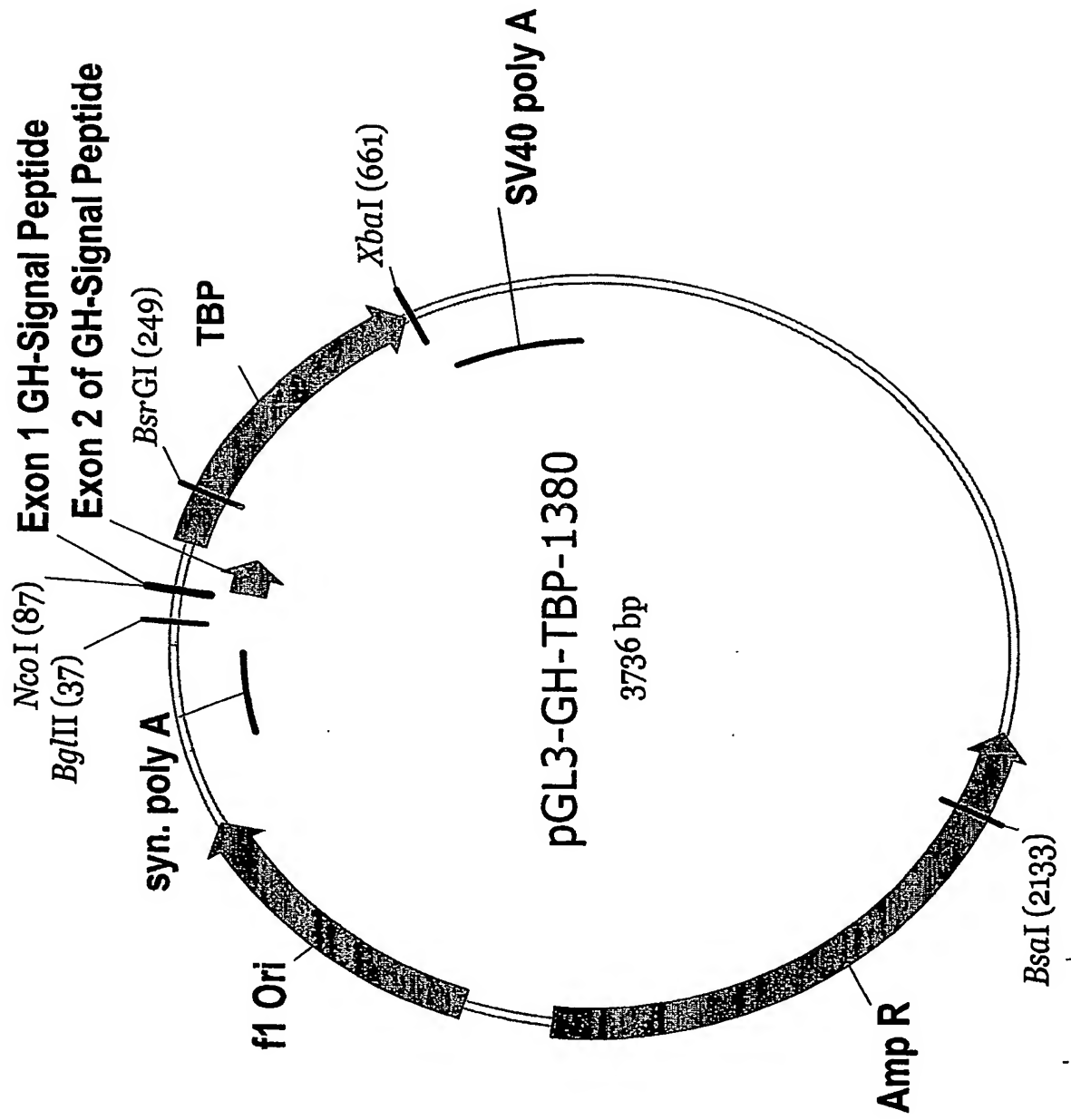


FIGURE 3

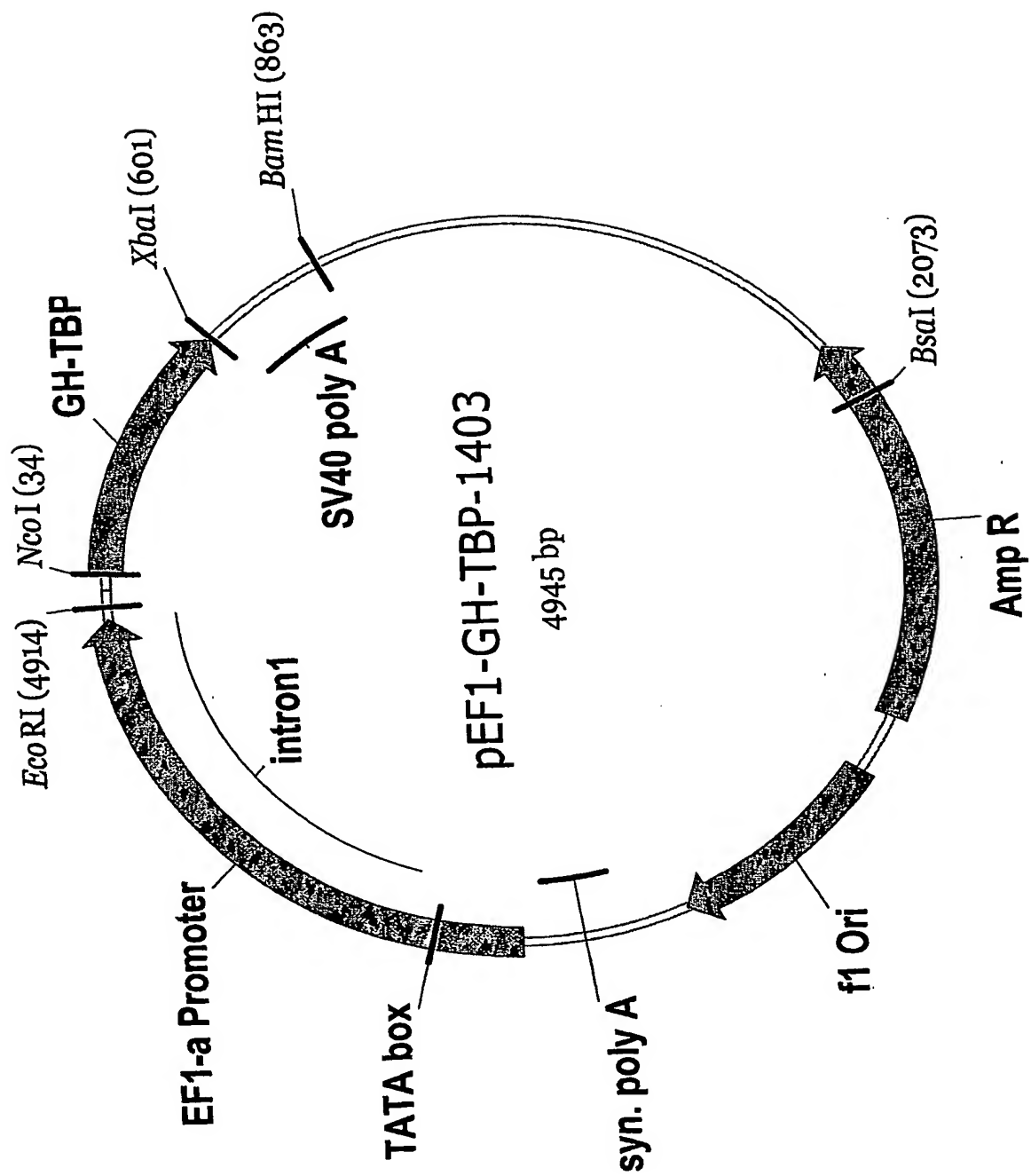


FIGURE 4

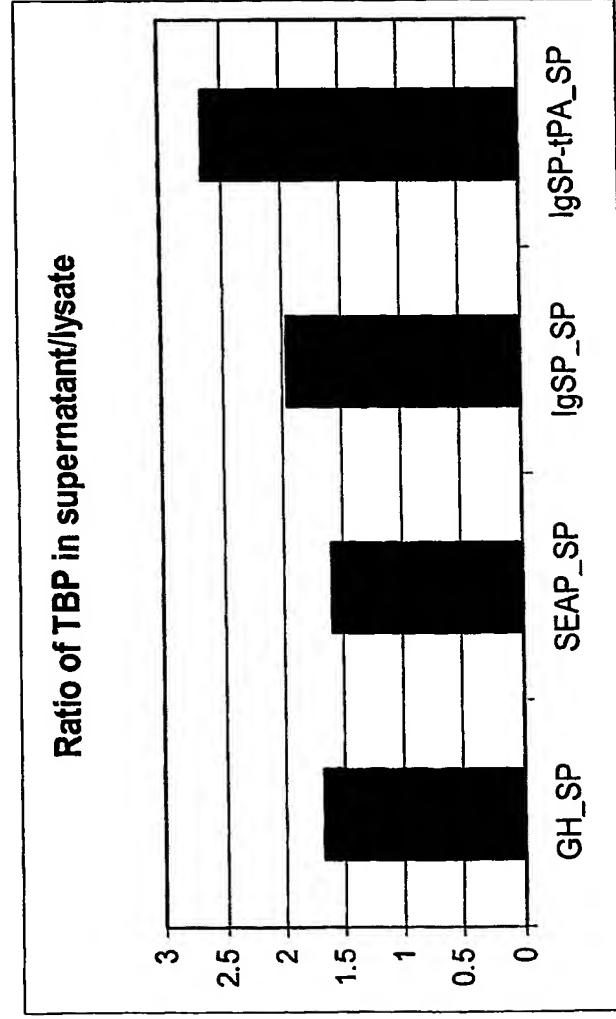


FIGURE 5

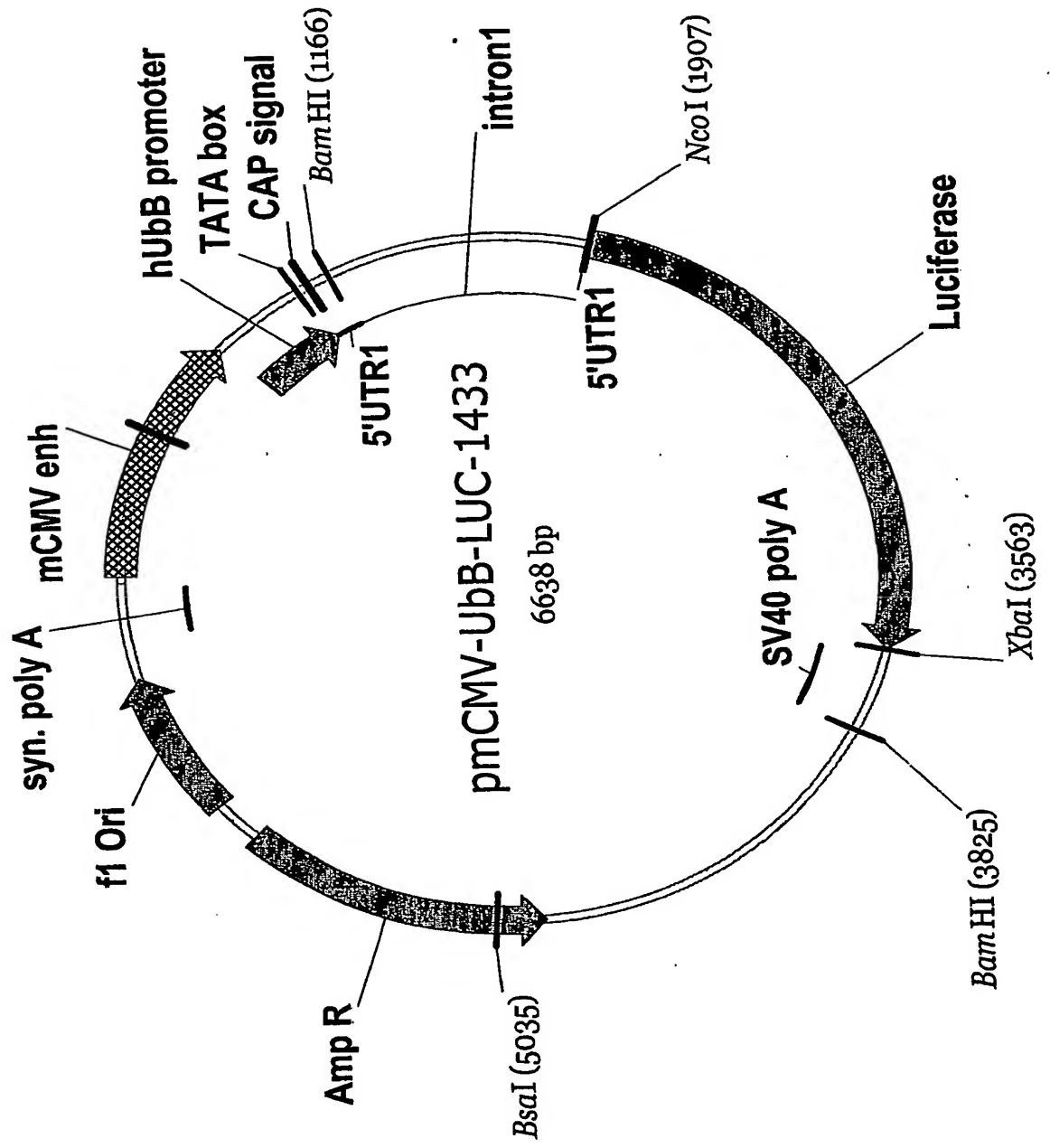
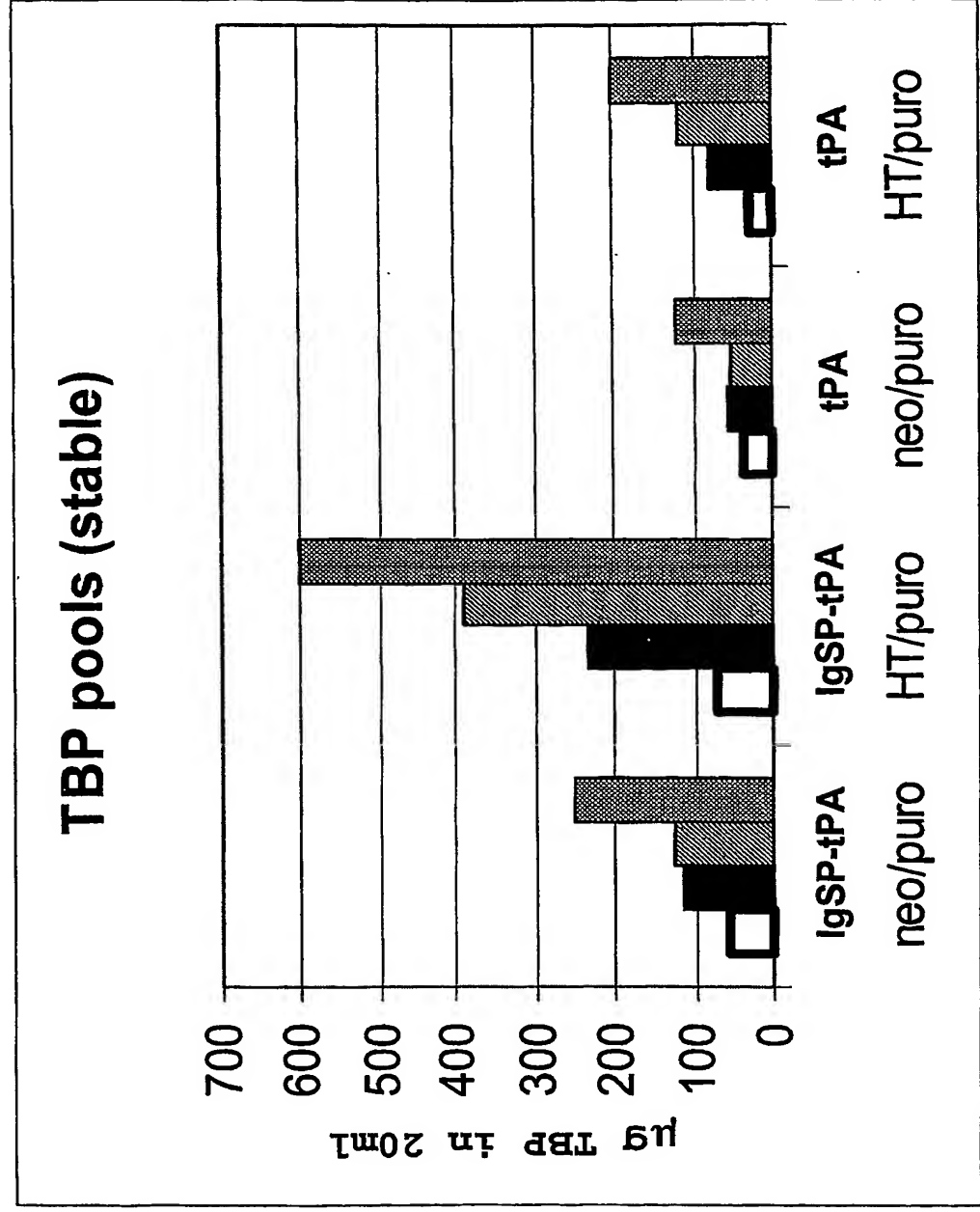


FIGURE 6



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